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Patentanmeldung Nr. Patent application No. Demande de brevet n°

02100334.8

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EPA/EPO/OEB Form 1014.1 - 02.2000 7001014

Anmeldung Nr:
Application no.: 02100334.8
Demande no:

Anmeldetag:
Date of filing: 03.04.02
Date de dépôt:

Anmelder/Applicant(s)/Demandeur(s):

Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
If no title is shown please refer to the description.
Si aucun titre n'est indiqué se referer à la description.)

NOVEL OX40R BINDING AGENTS

In Anspruch genommene Prioriät(en) / Priority(ies) claimed /Priorité(s) revendiquée(s)
Staat/Tag/Aktenzeichen/State/Date/File no./Pays/Date/Numéro de dépôt:

Internationale Patentklassifikation/International Patent Classification/
Classification internationale des brevets:

C07K14/00

Am Anmeldetag benannte Vertragstaaten/Contracting states designated at date of filing/Etats contractants désignées lors du dépôt:

AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE TR

NOVEL OX40R BINDING AGENTS

FIELD OF THE INVENTION

The present invention concerns novel peptides capable of modulating immune responses, in particular the ones under the control of OX40 Receptor - OX40 Ligand interaction.

BACKGROUND OF THE INVENTION

The cell regulatory system constituted by the membrane proteins OX40 Receptor (indicated in the literature also as OX-40, OX-40 Antigen, OX40R, TNFRSF4, and CD134) and OX40 Ligand (indicated in the literature also as OX40L, Glycoprotein GP34, ACT-4-L, TNFSF4, CD134 Ligand, and CD134L) has a prominent role in the regulation of immune responses as well as in the formation of secondary lymphoid tissue, similarly to other proteins belonging to the tumor necrosis factor ligand / receptor superfamilies (Gravestein and Borst, 1998; Weinberg, 2002). Many evidences have been provided by clinical observations and animal models, for example in gene targeting experiments (Chen et al., 1999; Kopf M et al., 1999; Murata K et al., 2000).

OX40 Receptor (OX40R, from now on) is a cell surface antigen, member of TNFR family, transiently expressed following T cell receptor (TCR) engagement and acting as a costimulatory receptor. It is considered as an highly specific $CD4^+$ or $CD8^+$ activation marker for T cells, being its expression strictly associated to therapeutically relevant tissue localizations, such as inflammation sites in multiple sclerosis, rheumatoid arthritis, as well as tumor-infiltrating lymphocytes and in the peripheral blood of animal models of graft-versus host disease.

OX40 Ligand (OX40L, from now on) is a transmembrane protein, originally identified as a protein stimulated by human T cell lymphotropic virus 1 (HTLV-1) infection and CD40 activation, having structural similarity to TNF and capable of forming cell-bound or secreted trimers. It is present on activated, antigen-presenting B and T cells, as well as dendritic cells, vascular endothelium cells and other non-hematopoietic tissues (heart, skeletal muscle, pancreas, testes and ovary). OX40L interacts with OX40R in the form of a homotrimer with a high affinity (Kd = 0.2-0.4 nM),

but no tridimensional structure has been solved so far to provide further molecular details.

The interaction between OX40L and OX40R has a co-stimulatory effect to OX40R-expressing effector T cells, leading to a more robust proliferative and cytokine responses due to the up-regulation of the cytokine production by T helper cells (Th1 and Th2) and to an increased survival of memory T cells through the inhibition of activation-induced cell death. Confirming evidences were also obtained in the transgenic mice lacking a functional OX40L gene, in autoimmunity animal models, where it was demonstrated that blocking the OX40R–OX40L interaction or depleting OX40R-positive T cells reduces clinical signs of autoimmunity, and in endothelium models, where OX40R–OX40L interaction appears to be involved in the control of activated T cells extravasation. Cumulatively, these expression and functional data raise the possibility that the signal transduction pathways regulated by OX40L-OX40R interaction may help to prolong antigen-specific proliferative responses or otherwise influence the persistence, differentiation or reactivation of effector / memory T cell populations.

The interest on OX40R-OX40L system is related to the fact that, even if the intracellular signaling mechanisms have not yet completely understood, the expression profile of OX40R makes this protein a peculiar target for $CD4^+$ T cells mediated diseases in clinical settings, for example in multiple sclerosis, where it is necessary to delete auto-reactive T cells. The hypothesis is that the products modulating OX40R activity may not have the serious side effects of conventional immunosuppressive therapies for autoimmune diseases and transplant rejection, which target all T cells.

The most studied approach for providing a therapeutic product based on OX40R-OX40L interaction makes use of OX40R binding agents in the form of antibodies against OX40R fused to toxins. These immunotoxins were able to decrease inflammation and ameliorate clinical signs in animal models for multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease, acute graft-versus-host disease.

Various OX40R binding agent have been disclosed in the prior art as having positive effects on immunization and cancer treatment (WO 99/42585; WO 95/21915; WO 95/21251; US 5457035). However, only large molecule such as the OX40L whole extracellular domain or antibodies are actually disclosed as being effective OX40R

binding agents. This also due to the fact that no real structure-activity studies have been performed to characterize this interaction, neither reliable information can be inferred from the analysis of other TNF / TNFR protein structures (Bodmer JL et al., 2002).

Since known OX40 binding agents proved to be useful as therapeutical and diagnostic agents, it would be desirable to identify compunds which, maintaining the binding and inhibiting property of known large molecules, are easier to generate and formulate such as peptides or other small molecules. Such interacting agents could be administered in a pharmaceutically acceptable form and in a therapeutically effective dosage for prophylaxis and therapy of pathological conditions mediated by activated T-cells, in particular $CD4^+$ T cells.

SUMMARY OF THE INVENTION

It has now been discovered that specific peptides derived fom the extracellular domain of OX40L can be used as OX40R binding agents. More specifically, it has been found that peptides corresponding to amino acids 94-124 of human OX40L, and in particular amino acids 107-116 of human OX40L, interact with human OX40R with high affinity, as shown by two different reliable screening technologies. These evidences can be exploited to use such peptides, heterologous proteins comprising theirsequences, as well as peptides and other molecules designed on their sequences, as OX40R binding agents. Compounds prepared in accordance with the present invention can be used to modulate, as OX40L agonists or antagonists, OX40R-mediated signaling in clinically relevant settings, thereby providing useful therapeutic compositions for use in the prophylaxis and/or treatment of diseases related to activated T-cells, in particular $CD4^+$ T cells. Other features and advantages of the invention will be apparent from the following detailed description.

DESCRIPTION OF THE FIGURES

Figure 1: (A) sequence of human OX40L, with the indication of the position of main protein domains (on the top of the sequence) and of the sequences corresponding to the peptides tested by Alphascreen™ (on the bottom of the

sequence); (B) inhibition of binding of human OX40R-Fc to human OX40L-CD8 by the peptides P4, P5, and a control without any peptide.

Figure 2: (A) sequence of peptide P5, with the indication of the sequence of the P5-derived peptides tested by Alphascreen™ (on the bottom of the sequence) and of the peptide P5-1a (boxed sequence in P5-1); (B) Inhibition of binding of human OX40R-Fc to human OX40L-CD8 by the peptide P5-1 compared with the effect provided by other fragments of peptide P5.

Figure 3: OX40R-Fc interaction with peptide P5 (A) and P5-1 (B), as measured by using fluorescence quenching spectroscopy. Non-linear regression analysis of OX40R fluorescence changes reveals a saturable binding with a dissociation constant for the OX40R / peptide complex indicated as Kd value for the two peptides.

DETAILED DESCRIPTION OF THE INVENTION

In view of the above mentioned evidences in the prior art, there is no indication of a specific sequence into OX40L extracellular domain which would be useful as OX40R binding agent for inhibiting OX40R-OX40L interaction.

By screening series of peptides derived from OX40L extracellular domain, amino acid sequences that interact with OX40R with high affinity were surprisingly identified and characterized as inhibitors of OX40R-OX40L interaction.

Accordingly, the present invention provides novel OX40R binding agent selected from:

a) polypeptides or peptides having the sequence corresponding to amino acids 94-124 (SEQ ID NO: 6), 107-116 (SEQ NO ID: 8), or 107-111 (SEQ ID NO: 13) of human OX40L;
b) active mutants of the polypeptides or peptides of (a) in which one or more amino acid residues have been added, deleted, or substituted;
c) peptide mimetics designed on the sequence and/or the structure of polypeptides or peptides of (a) or (b);
d) polypeptides or peptides comprising the amino acid sequence of (a) or (b), and an amino acid sequence belonging to a protein sequence other than human OX40L (SEQ ID NO: 1);

e) active fractions, precursors, salts, or derivatives of (a), (b), (c) or (d).

The amino acid sequence of OX40L (SEQ ID NO: 1) corresponds to the entry in the SWISSPROT database for human OX40L (P23510). The amino acid sequences listed under (a) have been shown, or inferred, to bind human OX40R protein, as shown in the examples of the present patent application. The activity was tested using a competition assay with recombinant forms of OX40L and OX40R, demonstrating that OX40L can be effectively competed by selected peptide sequences.

Fragments of the extracellular domain of ACT-4-L, which is an alternative name of OX40L, are disclosed as potential OX40R binding agents being associate to functional or structural domains in the extracellular domain of OX40L (WO 95/21915), but no evidence has been provided, herein or elsewhere in the art, on functional or structural domains of OX40L corresponding to the amino acids 94-124 (SEQ ID NO: 6), 107-116 (SEQ NO ID: 8), or 107-111 (SEQ ID NO: 13) of human OX40L (SEQ ID NO: 1). The present patent application successfully demontstrates that, even if no functional or structural domain can be inequivocally predicted by comparing other ligand/receptor pairs belonging to the same protein families (Bodmer JL et al., 2002), or by using well-known algorithms for protein structure prediction such as PREDATOR, PHD or HNN (accessiible, for example, at http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_server.html), such peptides are surprisingly effective as OX40R binding agents capable of inhibiting OX40R-OX40L interaction.

The term "peptide" is ordinarily applied to a polypeptidic chain containing from 4 to 30 or more contiguous amino acids, usually from 4 to 20 contiguous amino acids. Such peptides can be generated by methods known to those skilled in the art, including partial proteolytic cleavage of a larger protein, chemical synthesis, or genetic engineering.

The properties of amino acid sequences SEQ ID NO: 6, SEQ NO ID: 8, and SEQ ID NO: 13 can be maintained, or even potentiated, in their active mutants. This category of molecules includes natural or artificial analogs of said sequences, wherein one or more amino acid residues have been added, deleted, or substituted, provided they display the same biological activity characterized in the present invention at comparable or higher levels, as determined by means known in the art and dislcosed in the Examples below. Natural analogs are intended the corresponding sequences of

OX40L or OX40L-like proteins found in humans or in other organisms, like mouse OX40L (SWISSPROT Acc. N° P43488). Artificial analogs are intended peptides prepared by known chemical synthesis and/or by site-directed mutagenesis techniques, or any other known technique suitable thereof, which provide a finite set of substantially corresponding mutated or shortened peptides or polypeptides which can be routinely obtained and tested by one of ordinary skill in the art using the teachings presented in the prior art and in the Examples of the present paten t application.

In accordance with the present invention, preferred changes in these active mutants are commonly known as "conservative" or "safe" substitutions. Conservative amino acid substitutions are those with amino acids having sufficiently similar c hemical properties, in order to preserve the structure and the biological function of the molecule. It is clear that insertions and deletions of amino acids may also be made in the above defined sequences without altering their function, particularly if th e insertions or deletions only involve a few amino acids, e.g., under ten, and preferably under three, and do not remove or displace amino acids which are critical to the functional conformation of a protein or a peptide.

The literature provide many models on which the selection of conservative amino acids substitutions can be performed on the basis of statistical and physico-chemical studies on the sequence and/or the structure of natural protein (Rogov SI and Nekrasov AN, 2001). Protein design experiments have shown that the use of specific subsets of amino acids can produce foldable and active proteins, helping in the classification of amino acid "synonymous" substitutions which can be more easily accommodated in protein structure, and which can be used t o detect functional and structural homologs and paralogs (Murphy LR et al., 2000). The synonymous amino acid groups and more preferred synonymous groups are those defined in Table I.

Active mutants produced by substitutions made on the basis of these teach ings, as well as active mutants wherein one or more amino acids were eliminated or added, are amongst the objects of the present invention, that is, novel polypeptides or peptides having the OX40L binding activity and OX40R-OX40L interaction inhibiting activity, comparable to the ones of selected peptides, or even improved if possible.

The above described alternative compounds term are intended to comprehend molecules with changes to the sequence of the OX40 protein which do not affect the

basic characteristics disclosed in the present patent application, particularly insofar as its ability of binding and inhibiting OX40R is concerned. Similar compounds may result from conventional mutagenesis technique of the encoding DNA, from combinatorial technologies at the level of encoding DNA sequence (such as DNA shuffling, phage display/selection), or from computer-aided design studies, followed by the screening for the desired activity as described in the prior art and in the Examples below.

In particular, the invention provides novel OX40R binding agents consisting of peptide mimetics (also called peptidomimetics) of SEQ ID NO: 6, SEQ NO ID: 8, and SEQ ID NO: 13, in which the nature of peptide or polypeptide has been chemically modified at the level of amino acid side chains, of amino acid chirality, and/or of the peptide backbone. These alterations are intended to provide OX40R binding agents compounds having similar or improved therapeutic, diagnostic and/or pharmacokinetic properties.

For example, when the peptide is susceptible to cleavage by peptidases following injection into the subject is a problem, replacement of a particularly sensitive peptide bond with a non-cleavable peptide mimetic can provide a peptide more stable and thus more useful as a therapeutic. Similarly, the replacement of an L-amino acid residue is a standard way of rendering the peptide less sensitive to proteolysis, and finally more similar to organic compounds other than peptides. Also useful are amino-terminal blocking groups such as t-butyloxycarbonyl, acetyl, theyl, succinyl, methoxysuccinyl, suberyl, adipyl, azelayl, dansyl, benzyloxycarbonyl, fluorenylmethoxycarbonyl, methoxyazelayl, methoxyadipyl, methoxysuberyl, and 2,4,-dinitrophenyl. Many other modifications providing increased potency, prolonged activity, easiness of purification, and/or increased half-life are known in the art (WO 02/10195; Villain M et al., 2001). Preferred alternative, "synonymous" groups for amino acids included in peptide mimetics are those defined in Table II.

The techniques for the synthesis and the development of peptide mimetics, as well as non-peptide mimetics, are well known in the art (Sawyer TK, 1997; Hruby VJ and Balse PM, 2000; Golebiowski A et al., 2001; Kim HO and Kahn M, 2000). Various methodology for incorporating unnatural amino acids into proteins, using both *in vitro* and *in vivo* translation systems, to probe and/or improve protein structure and function are also disclosed in the literature (Dougherty DA, 2000).

Other OX40R binding agents can be peptide or non-peptide mimetics identified by methods of computer-aided drug design which make use of SEQ ID NO: 6, SEQ NO ID: 8, or SEQ ID NO: 13 amino acid sequences. The tridimensional structure of OX40L and OX40R, as separate molecules or as a complex, has not been solved yet but the disclosure provided in this patent application, once that this information will be available, will probably allow to study the interaction between OX40L and OX40R with greater efficacy using these and other simulation technologies (Cochran A et al., 2001; Kraemer-Pecore CM et al., 2001). Such computer-assisted analysis can be exploited to develop improved peptide or non-peptide mimetic drugs in the form of synthetic organic molecules or peptides (for example, having between 4 and 20 amino acid). Once that these compounds have been screened and found to be capable of binding OX40R and competing with OX40L, it will then be assessed their utility using cell or animal models.

The present patent application discloses as OX40 binding agents polypeptides or peptides comprising the amino acid sequence as defined above in (a) or (b), and an amino acid sequence belonging to a protein sequence other than human OX40L. This heterologous latter sequence should provide additional properties without impairing significatively OX40R binding activity. Examples of such additional properties are an easier purification procedure, a longer lasting half-life in body fluids, or extracellular localization. This latter feature is of particular importance for defining a specific group of fusion or chimeric proteins included in the above definition since it allows the peptides characterized as OX40R binding agent in this patent application to be localized in the space where not only where the isolation and purification of these peptides is facilitated, but also where OX40L and OX40R naturally interact.

Additional protein sequences which can be used to generate the polypeptide or peptide of (d) are the ones of membrane-bound proteins, extracellular domains of membrane-bound protein, immunoglobulin constant region, multimerization domains, extracellular proteins, signal peptide-containing proteins, export signal-containing proteins. The choice of one or more of these sequences to be fused to the OX40 binding agent is functional to specific use of said agent. As a general procedure, these fusion proteins can be produced by generating nucleic acid segments encoding them, using common genetic engineering techniques, and cloning in replicable vector of viral or plasmid origin which are used to modify a Prokaryotic or Eukaryotic host cell, using

episomal or non-/homologously integrated vectors, as well as transformation-, infection-, or transfection-based technologies. These vectors should allow the expression of the fusion protein including the OX40R binding agent in the prokaryotic or eukaryotic host cell under the control of their own transcriptional initiation/termination regulatory sequences, which are chosen to be constitutively active or inducible in said cell. A cell line substantially enriched in such cells can be then isolated to provide a stable cell line. In particular, whenever the cells modified to express the OX40R binding agents of the invention are directly used or administered, preferred cells are human cells normally expressing OX40L, in particular human B cells.

When the additional protein sequence, as in the case of the sequence of extracellular, export signal, or signal-peptide containing proteins, allows the OX4OR binding domain to be secreted in the extracellular space, the agent can be more easily collected and purified from cultured cells in view of further processing or, alternatively, the cells can be directly used or administered.

When the additional protein, as in the case of the sequence of membrane-bound proteins, allows the immobilization of the OX40R binding agent on the surface of the cell, the agent can be less easily collected and purified from the cultured cells in view of further processing but the cells can be directly used or administered providing the agent in a form corresponding to the one of natural OX40L, possibly improving its properties.

Finally, since OX40L-OX40R interaction is known to involve multimerization of the proteins, in particular the trimerization (Al Shamkhani A et al., 1997). Therefore, the fusion protein may also include sequence allowing the multimerization of the resulting protein, such as the immunoglobulin constant regions, extracellular domains of membrane-bound proteins, or trimerization domains known in the art as being present in TNFR-like (WO 00/39295) or other proteins (WO 01/49866, WO 99/10510, WO 01/98507). Other useful protein sequences that can be included are the ones providing means of purification by affinity chromatography (Constans A, 2002; Lowe CR et al., 2001).

The polypeptides and the peptides of the present invention can be in other alternative forms which can be preferred according to the desired method of use and/or

production, for example as active fractions, precursors, salts, derivatives, conjugates or complexes.

The term "active" means that such alternative compounds should maintain the functional features of the OX40R binding agent of the present invention, and should be as well pharmaceutically acceptable and useful.

The term "fraction" refers to any fragment of the polypeptidic chain of the compound itself, alone or in combination with related molecules or residues bound to it, for example residues of sugars or phosphates, or aggregates of the original polypeptide or peptide. Such molecules can result also from other modifications which do not normally alter primary sequence, for example *in vivo* or *in vitro* chemical derivativization of peptides (acetylation or carboxylation), those made by modifying the pattern of phosphorylation (introduction of phosphotyrosine, phosphoserine, or phosphothreonine residues) or glycosylation (by exposing the peptide to enzymes which affect glycosylation e.g., mammalian glycosylating or deglycosylating enzymes) of a peptide during its synthesis and processing or in further processing steps. For example, SEQ ID NO: 6 and SEQ ID NO: 8 contain a potential glycosylation site (amino acids 114-116 in human OX40L) and this can be modified accordingly during the recombinant expression in the host cell or during chemical synthesis.

The "precursors" are compounds which can be converted into the compounds of present invention by metabolic and enzymatic processing prior or after the administration to the cells or to the body.

The term "salts" herein refers to both salts of carboxyl groups and to acid addition salts of amino groups of the peptides, polypeptides, or analogs thereof, of the present invention. Salts of a carboxyl group may be formed by means known in the art and include inorganic salts, for example, sodium, calcium, ammonium, ferric or zinc salts, and the like, and salts with organic bases as those formed, for example, with amines, such as triethanolamine, arginine or lysine, piperidine, procaine and the like. Acid addition salts include, for example, salts with mineral acids such as, for example, hydrochloric acid or sulfuric acid, and salts with organic acids such as, for example, acetic acid or oxalic acid. Any of such salts should have substantially similar activity to the peptides and polypeptides of the invention or their analogs.

The term "derivatives" as herein used refers to derivatives which can be prepared from the functional groups present on the lateral chains of the amino acid moieties or on the N-/ or C-terminal groups according to known methods. Such derivatives include for example esters or aliphatic amides of the carboxyl-groups and N-acyl derivatives of free amino groups or O-acyl derivatives of free hydroxyl-groups and are formed with acyl-groups as for example alcanoyl- or aroyl-groups.

Useful conjugates or complexes of the OX40R binding agents of the present invention can be generated, using molecules and methods known in the art (as shown for anti-OX40R antibodies in WO 95/21251) to improve the detectability of the interaction with OX40R (radioactive or fluorescent labels, biotin), therapeutic efficacy (cytotoxic agents), or improving the agents in terms of drug delivery efficacy, such as polyethylene glycol and other natural or synthetic polymers (Pillai O and Panchagnula R, 2001).

The compounds of the invention may be prepared by any well known procedure in the art, including recombinant DNA-related technologies described above, and chemical synthesis technologies.

Examples of chemical synthesis technologies are solid phase synthesis and liquid phase synthesis. As a solid phase synthesis, for example, the amino acid corresponding to the C-terminus of the peptide to be synthetized is bound to a support which is insoluble in organic solvents, and by alternate repetition of reactions, one wherein amino acids with their amino groups and side chain functional groups protected with appropriate protective groups are condensed one by one in order from the C-terminus to the N-terminus, and one where the amino acids bound to the resin or the protective group of the amino groups of the peptides are released, the peptide chain is thus extended in this manner. Solid phase synthesis methods are largely classified by the tBoc method and the Fmoc method, depending on the type of protective group used. Typically used protective groups include tBoc (t-butoxycarbonyl), Cl-Z (2-chlorobenzyloxycarbonyl), Br-Z (2-bromobenzyloxycarbonyl), Bzl (benzyl), Fmoc (9-fluorenylmethoxycarbonyl), Mbh (4,4'-dimethoxydibenzhydryl), Mtr (4-methoxy-2,3,6-trimethylbenzenesulphonyl), Trt (trityl), Tos (tosyl), Z (benzyloxycarbonyl) and Cl2-Bzl (2,6-dichlorobenzyl) for the amino groups; NO2 (nitro) and Pmc (2,2,5,7,8-pentamethylchromane-6-sulphonyl) for the guanidino

groups); and tBu (t-butyl) for the hydroxyl groups). After synthesis of the desired peptide, it is subjected to the de-protection reaction and cut out from the solid support. Such peptide cutting reaction may be carried with hydrogen fluoride or tri-fluoromethane sulfonic acid for the Boc method, and with TFA for the Fmoc method.

The OX40R binding agents obtained by recombinant DNA- or chemical synthesis technologies are finally subjected to one or more steps of purification. Purification can be carried out by any one of the methods known for this purpose, i.e. any conventional procedure involving extraction, precipitation, chromatography, electrophoresis, or the like. For example, HPLC (High Performance Liquid Chromatography) can be used. The elution can be carried using a water-acetonitrile-based solvent commonly employed for protein purification. The invention includes purified preparations of the compounds of the invention. Purified preparations, as used herein, refers to the preparations which are at least 1%, preferably at least 5%, by dry weight of the compounds of the invention.

The compounds of the invention described above (proteins, peptides, organic compounds) can be used as the active ingredients in pharmaceutical compositions for the prophylaxis and/or treatment of diseases related to the activation of $CD4^+$ T cells, the cells where OX40R-OX40L interactions have been characterized as being important signaling events. Therefore, novel methods for treating a patient suffering from a condition mediated by $CD4^+$ T cells can comprise the administration to the patient an effective amount of an OX40R binding agent, in the form of above described compounds or cells.

The OX40R binding agents, as well the cells expressing them, disclosed in the present patent application have not been tested yet as either agonist or antagonist of OX40L on cells expressing OX40R on their surface. However, in both cases, the utility of these molecules can be predicted, before performing any functional assay in animal models, on the basis of prior art on cell based assay for simulating specific physiological events specifically associated to OX40L and OX40R activity and / or other metabolic and physiological events (Coleman RA et al., 2001; Obinata M, 2001)

In the case that an OX40R binding agent of the invention, once bound to OX40R, acts as agonist of the competed OX40L, the therapeutical potential of such molecule would be in the prophylaxis and/or treatment of transplantation rejection or cancer,

where a stimulation of $CD4^+$ T cells activation is beneficial. In the case that an OX40R binding agent of the invention, once bound to OX40R, acts as antagonist of the competed OX40L, the therapeutical potential of such molecule would be in the prophylaxis and/or treatment autoimmune diseases (e.g. inflammatory bowel disease, rheumatoid arthritis, and multiple sclerosis), inflammations or infections, where an inhibition of $CD4^+$ T cells activation is beneficial. This latter effect can be also used for reducing the population of $CD4^+$ T cells that express OX40R.

The agonistic or antagonistic effects associated to OX40R binding agents can be also attributed to the cell lines expressing such OX40R binding agents when used or adminstered directly. Moreover, the $CD4^+$ T cells to which the agents are directed to can be comprised in an animal, when the use is applied *in vivo*, or in an organ, in a tissue, or in cultured cells, when the use is performed *ex vivo*, that is, when have been extracted from the body and kept outside for a short period to provide a specific therapeutic treatment before being implanted again in the body.

The present invention also provides pharmaceutical compositions comprising one of the OX40R binding agents of the invention as active ingredient for the prophylaxis and/or treatment of diseases related to $CD4^+$ T cells. These pharmaceutical compositions can be formulated in combination with pharmaceutically acceptable carriers, excipients, stabilizers, or diluents. Depending on the properties of the agent, the pharmaceutical composition can be useful for diseases related to $CD4^+$ T cells such transplant rejection, autoimmune diseases, inflammation, infection, or cancer. The specific therapeutic potential of such compositions depends on the antagonistic or agonistic properties of the specific OX40R binding agent used as active ingredient, as defined before.

Pharmaceutical compositions comprising the OX40R-OX40L interaction inhibitory compounds of the present invention include all compositions wherein said compound is contained in therapeutically effective amount, that is, an amount effective to achieve the medically desirable result in the treated animal. The pharmaceutical compositions may contain suitable pharmaceutically acceptable carriers, biologically compatible vehicles which are suitable for administration to an animal (for example, physiological saline) and eventually comprising auxiliaries (like excipients, stabilizers or diluents)

which facilitate the processing of the active compounds into preparations which can be used pharmaceutically.

The pharmaceutical compositions may be formulated in any acceptable way to meet the needs of the mode of administration. The use of biomaterials and other polymers for drug delivery, as well the different techniques and models to validate a specific mode of administration, are disclosed in literature (Luo B and Prestwich GD, 2001; Cleland JL et al., 2001). Modifications of the compounds of the invention to improve penetration of the blood-brain barrier would also be useful. Other methods of biomimetic transport and rational drug delivery in the field of transvascular drug delivery are known in the art (Ranney DF, 2000).

Any accepted mode of administration can be used and determined by those skilled in the art. For example, administration may be by various parenteral routes such as subcutaneous, intravenous, intradermal, intramuscular, intraperitoneal, intranasal, transdermal, or buccal routes. Parenteral administration can be by bolus injection or by gradual perfusion over time. Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions, which may contain auxiliary agents or excipients known in the art, and can be prepared according to routine methods. In addition, suspension of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or triglycerides. Aqueous injection suspensions that may contain substances increasing the viscosity of the suspension include, for example, sodium carboxymethyl cellulose, sorbitol, and/or dextran. Optionally, the suspension may also contain stabilizers. Pharmaceutical compositions include suitable solutions for administration by injection, and contain from about 0.01 to 99 percent, preferably from about 20 to 75 percent of active compound together with the excipient. Compositions which can be administered rectally include suppositories.

It is understood that the dosage administered will be dependent upon the age, sex, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired. The dosage will be tailored to the individual subject, as is understood and determinable by one of skill in the art. The total

dose required for each treatment may be administered by multiple doses or in a single dose. The pharmaceutical composition of the present invention may be administered alone or in conjunction with other therapeutics directed to the condition, or directed to other symptoms of the condition. Usually a daily dosage of active ingredient is comprised between 0.01 to 100 milligrams per kilogram of body weight.

The compounds of the present invention may be administered to the patient intravenously in a pharmaceutically acceptable carrier such as physiological saline. Standard methods for intracellular delivery of peptides can be used, e.g. delivery via liposomes. Such methods are well known to those of ordinary skill in the art. The formulations of this invention are useful for parenteral administration, such as intravenous, subcutaneous, intramuscular, and intraperitoneal.

As well known in the medical arts, dosages for any one patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently.

The present invention provides novel means for modulating an immune response by altering OX40R-OX40L interaction *in vivo* or *ex vivo,* represented by the disclosed OX40R binding agents, and the corresponding nucleic acid and expressing cells, but provides also a series of novel applications which derive from the efficient binding of OX40R, independently from the agonistic or antagonistic properties.

The OX40R binding agents and the OX40R binding agent-expressing cells of the present patent application can be used for the detection of the extracellular domain of OX40R protein, as membrane-bound or a soluble protein. Obviously this use can be extended to the detection of activated $CD4^+$ T cells expressing OX40R protein.

The method of detections based on these uses comprises a first step in which a sample is contacted with the agents or the cells, and a second step in which the interaction between the extracellular domain of OX40R protein is detected directly (by the means of any label associated to the agent or the cell, as described before) or indirectly (by the means of the effects of this binding on the OX40R protein or the OX40R-expressing cell, for example), in order to indicate the presence of these elements.

The agent or the cells can be immobilized, before or after being put in contact with the sample, onto supports which allow not only the detection but also the purification, and / or the concentration of the OX40R extracellular domain, as membrane-bound or a soluble protein, or OX40R-expressing cells. These supports can be consequently used in methods for the detection, the purification, and / or the concentration of OX40R extracellular domain, as membrane-bound or a soluble protein, or OX40R-expressing cells in a sample by contacting said sample with the supports. This and the previously described detection methods can be used to diagnose a condition associated to decreased or increased presence of $CD4^+$ T cells or of soluble OX40R protein.

The present invention also discloses screening assay for the determination of the nature and the activity of compounds, as provided in Examples of the present patent application, inhibiting OX40R-OX40L interactions comprising:

a) Forming a sample comprising the following elements:
   i. An element constituting the OX40R binding agent,, chosen amongst the peptides, the compounds, the cells, and the supports described in the present invention;
   ii. An element constituting the OX40R moiety, chosen amongst a protein comprising the extracellular domain of OX40R, a cell line expressing OX40R extracellular domain on its surface, and a cell line secreting extracellular domain of OX40R; and
   iii. The compound(s) to be tested as inihibitor(s) of OX40R-OX40L interaction.
b) Detecting, directly or indirectly, the effect of the compounds (iii) on the interactions between the elements (i) and (ii).
c) Comparing the effect detected in (b) amongst samples different in terms of quality and / or quantity of the elements of (a).

The support disclosed above represents a preferable element since those kinds of screening are more efficient and quick by using an binding element immobilized on supports like plastic microtiter plate or beads.

Finally, the present invention also provides novel kits comprising the OX40R binding agents, or the corresponding cells or supports here disclosed, for detecting

extracellular domain of OX40R protein (as membrane-bound or a soluble protein), and activated CD4$^+$ T cells, allowing also the diagnosis of a condition due to a decreased or increased presence of CD4$^+$ T cells or of soluble OX40R protein in a sample obtained from a patient. Kits comprising such agents, cells or supports, can be also used for determining the nature and the activity of compounds inhibiting OX40R-OX40L interaction.

All references cited herein are entirely incorporated by reference herein, including all data, tables, figures, and text presented in the cited references. Additionally, the entire contents of the references cited within the references cited herein are also entirely incorporated by reference. Reference to known method steps, conventional method steps, known methods or conventional methods is not in any way an admission that any aspect, description or embodiment of the present invention is disclosed, taught or suggested in the relevant art. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Once understood the features of the methods and products disclosed in present application, the necessity and kind of additional steps can be easily deduced by reviewing prior art, as well as the non-limiting following figures and examples describing the basic details and some applications of the invention.

EXAMPLES

<u>Example 1</u>: Identification of peptides derived from human OX40L interacting with human OX40R using AlphaScreen™ Assay.

<u>Methods</u>

Peptides and proteins

Recombinant human OX40R-Fc fusion protein was prepared as a fusion protein containing the extracellular portion of OX40 fused to the Fc region of IgG. HEK293 EBNA cells were transfected, using the calcium phosphate technique, with an expression construct based on pcDNA3 plasmid (Invitrogen) in which the coding sequence for the N-terminal, extracellular domain of OX40R (amino acids 1-212 of SWISSPROT Acc. N° P43489) is put in 5' to the coding sequence for C-terminal hinge/CH2/CH3 domains of human Ig gamma 1C (amino acids 98-330 of SWISSPROT Acc. N° P01857). The resulting recombinant fusion protein is expressed by the host

cells as a secreted protein due to the signal sequence of OX40R, together with a selectable marker gene for Hygromycin B.

The culture supernatant was clarified by centrifugation (10 minutes at 500x g) and subsequently filtered using PVDF membranes of 0.45 and 0.22 micrometrs pore size (Millipore). In parallel, the purification column, containing 38 ml resin on which recombinant protein G was immobilized (Pharmacia), was equilibrated in loading buffer (0.1 M Tris pH 7.0) using a Bio-Logic FPLC system (Biorad). After equilibration with 20 column-volumes (CV) of loading buffer, the sample was applied to the column at a flow rate of 1ml/min. The column was washed away with a 10 CV of loading buffer to eliminate protein bound unspecifically to the resin. OX40-Fc fusion protein, immobilized on the protein G via the Fc moiety, was eluted by a step gradient using a Glycine/HCl (pH 3.0) elution buffer. The resulting fractions were directly neutralized with 1M Tris pH 7.6 to prevent protein degradation in the acidic elution buffer. Finally, the fractions containing the OX40R-Fc fusion protein were desalted using a Sephadex G25 column (Pharmacia) equilibrated in PBS (Phosphate Buffer Saline).

Human OX40L-CD8 and anti-CD8 biotinylated antibodies are commercially available (Ancell). The sOX40L-GST was prepared as a fusion protein by cloning the extracellular portion of OX40L fused to GST in a plasmid then used to transfect SF9 cells, cultivated in SF900 II media (Invitrogen).

The peptides were synthesized at purity ranging between 85-97% by Synten (France), and stored in lyophilized form at –20°C. The peptides were solubilised in 4% NaOH 1M in PBS before use. The name, sequence, and the corresponding amino acids in human OX40L for each peptides is shown in Table III.

Alphascreen™ Assay

Alphascreen™ acceptor and donor beads were purchased (Alphascreen™; Packard BioScience): The OX40R-Fc chimeric protein was immobilized onto protein A-conjugated acceptor beads. The OX40L-CD8 chimeric protein was immobilized, by the means of a biotinylated anti-CD8 antibody, onto streptavidin-conjugated donor beads.

The assay was performed by mixing 5 microliters of OX40R-Fc (10 nM), 5 microliters of OX40L-CD8 (10 nM), 5 microliters of biotinylated anti-CD8 (10 nM), 5 microliters of Streptavidin donor beads (20 mg/ml) and 5 microliters Protein A acceptor

beads (20mg/ml) were mixed in a each well of a 384 well plate. The plate was read at an excitation wavelength of 680 nanometers combined with a shorter emission wavelength of 520-620 nanometers.

The same components were mixed at the same concentration as described before for the competition assays, wherein various concentrations of each peptide were added to the reaction in a 5 microliter volume. The plates were incubated for 2 hours in the dark, at room temperature, and with shaking. The plate was read on a Fusion Instrument at a long excitation wavelength of 680 nm combined with a shorter emission wavelength of 520-620 nanometers.

<u>Results</u>

A screening system to establish the potential inhibiting properties of OX40L-derived peptides on the OX40R/OX40L interaction was set up by making use of a commercially available technology called Amplified Luminescent Proximity Homogeneous Assay Screen (Alphascreen™; Packard Bioscience), a method based on the Luminescent Oxygen Channeling Immunoassay (LOCI; EP515194; Ullman E et al., 1994).

Briefly, the principle of AlphaScreen™ technology provides an easy and reliable determination of the effect of compounds on biomolecular interactions and activities, in particular for protein/protein interaction assays. AlphaScreen relies on the use of "Donor" and "Acceptor" beads, each coated with a layer of hydrogel providing functional groups for conjugation of a specific molecule. When a biological interaction between the immobilized molecules brings the beads into proximity, a cascade of chemical reactions is initiated to produce a greatly amplified signal. Upon laser excitation, a photosensitizer in the "Donor" bead converts ambient oxygen to a more excited singlet state. The excited singlet state oxygen molecules diffuse across a short distance and decay to react with a chemiluminescer contained within the Acceptor bead. This fluorophore subsequently emits light at 520-620 nanometers. In the absence of a specific biological interaction, the singlet state oxygen molecules produced by the Donor bead go undetected without the close proximity of the Acceptor bead. AlphaScreen™ technology allows the detection of interactions with affinities in the sub-nanomolar / micromolar range.

The experimental set-up was first tested using only the basic binding partners, immobilized on AlphaScreen™ beads by making use of different affinity tags. The

binding and dissociation of OX40L-CD8 with OX40-Fc kinetics were analysed using a range of OX40L-CD8 concentrations giving an apparent KD of ~7 nM. The specificity of the interaction was demonstrated by using sOX40L-GST which inhibits the binding of OX40-Fc receptor to the OX40L-CD8 with an IC_{50} of 2 µM.

Two series of peptides were designed on the sequence of extracellular domain of human OX40L, which corresponds to the amino acids 51-183 of the protein according to the SWISSPROT record (P23510). Using a first series of peptides (figure 1A), it was possible to define a first minimal peptide (P5) capable to inhibit the binding of OX40R to OX40L in the micromolar range (figure 1B), an affinity value which is pharmacologically relevant.

On the basis of the sequence of the P5 peptide, a second series of peptides were tested to further reduce this inhibiting molecule (figure 2A). The result of this sequential screening is that the region comprised between amino acids 94 and 124 of human OX40L (P5) is not a minimal region since a peptide corresponding to amino acids 107 - 116 (P5-1), is capable to inhibit the binding of OX40R to OX40L in the micromolar range (Kd ~10 and 62 microMolar respectively). The other tested peptides showed no or hardly measurable effect on OX40R-OX40L interaction (figure 2B). Since the P4 peptide, which contains the C-terminal six amino acids of P5-1 peptide, proved to bind OX40R very poorly, it can be also inferred that the N-terminal amino acids of P5-1 peptide, for example the sequence GYFSQ (peptide P5-1a; amino acids 107–111 in human OX40L; SEQ ID NO: 13), may represent a minimal peptide sequence functionally active as OX40R binding agent.

The sequences of P5 and P5-1 peptides identified in this example by competition assays allowed to identify structures in the OX40L which play an essential role in the OX40L-OX40R interaction, demonstrating that OX40L can be effectively competed by the selected peptide sequences. These findings were not predictable from the analysis of the state of the art on the structure-activity relationship of these proteins, neither of other TNF / TNFR- like proteins, being the regions of contact between proteins belonging to these families very diverse amongst the pairs of ligands and receptors (Bodmer JL et al., 2002).

<u>Example 2</u>: **Affinity of P5 and P5-1 peptides for human OX40R in solution**

measured by the fluorescence quenching spectroscopy.

Methods

Fluorescence quenching Assay

The fluorescence quenching assay was performed as previously described (Golabek A et al., 2000). OX40R-Fc protein (35 micrograms) was dissolved in 500 microliters of PBS and the fluorescence spectum was recorded at 295-420 nanometers with an excitation wavelength of 290 nanometers using a spectrofluorimeter (Perkin Elmer LS50B) with the slits set at 5 nanometers. Fluorescence spectra of human OX40R-Fc were then recorded in the presence of increasing concentrations (5-1000 nM) of P5 and P5-1 peptides after 15 minutes of equilibration. The fluorescence change at 336 nanometers was plotted versus peptide concentration and the resulting curves were analyzed by nonlinear regression fit with Prism® software (GraphPad).

Results

The affinity of the peptides identified in Example 1 as inhibitor of OX40L/OX40R interaction was evaluated by fluorescence quenching spectroscopy, a technology which allows such measurements in solution, under native conditions and without beads or other supports. This method is based on monitoring the changes in the intrinsic fluorescence of a protein (OX40R-Fc) upon its binding with another protein (P5 or P5-1). Incubation of OX40R-Fc with increasing concentrations of P5 or P5-1 peptides caused a change of its intrinsic fluorescence in the form of an hyperbolic curve, when the changes in OX40-Fc fluorescence were plotted against peptide P5 and P5-1 concentration (Figure 3A and 3B). Nonlinear regression analysis of the data revealed an apparent dissociation constant for OX40R/P5 complex of KD ~7.9 nM, and for OX40/P5-1 complex of KD was ~24.6 nM. This values are considered revealing of high affinity interaction

The findings presented in the Examples indicate that OX40R-OX40L interaction can be effectively inhibited by using specific OX40L-derived peptides which bind OX40R with high affinity, providing novel opportunity for the development of drugs targeting OX40R pathway and controlling aberrant or undesirable physiological events under its control. It is possible to further characterize the OX40R binding agents disclosed in this patent application, as agonist or antagonist of OX40L in its interaction with OX40R, using the animal and cell biology assay known in the art (WO 99/42585;

WO 95/21915, WO 95/21251; Imura A et al., 1997; Nohara C et al., 2001; Pippig SD et al., 1999).

TABLE I

Amino Acid	Synonymous Group	More Preferred Synonymous Groups
Ser	Gly, Ala, Ser, Thr, Pro	Thr, Ser
Arg	Asn, Lys, Gln, Arg, His	Arg, Lys, His
Leu	Phe, Ile, Val, Leu, Met	Ile, Val, Leu, Met
Pro	Gly, Ala, Ser, Thr, Pro	Pro
Thr	Gly, Ala, Ser, Thr, Pro	Thr, Ser
Ala	Gly, Thr, Pro, Ala, Ser	Gly, Ala
Val	Met, Phe, Ile, Leu, Val	Met, Ile, Val, Leu
Gly	Ala, Thr, Pro, Ser, Gly	Gly, Ala
Ile	Phe, Ile, Val, Leu, Met	Ile, Val, Leu, Met
Phe	Trp, Phe,Tyr	Tyr, Phe
Tyr	Trp, Phe,Tyr	Phe, Tyr
Cys	Ser, Thr, Cys	Cys
His	Asn, Lys, Gln, Arg, His	Arg, Lys, His
Gln	Glu, Asn, Asp, Gln	Asn, Gln
Asn	Glu, Asn, Asp, Gln	Asn, Gln
Lys	Asn, Lys, Gln, Arg, His	Arg, Lys, His
Asp	Glu, Asn, Asp, Gln	Asp, Glu
Glu	Glu, Asn, Asp, Gln	Asp, Glu
Met	Phe, Ile, Val, Leu, Met	Ile, Val, Leu, Met
Trp	Trp, Phe,Tyr	Trp

TABLE II

Amino Acid	Synonymous Group
Ser	D-Ser, Thr, D-Thr, allo-Thr, Met, D-Met, Met(O), D-Met(O), L-Cys, D-Cys
Arg	D-Arg, Lys, D-Lys, homo-Arg, D-homo-Arg, Met, Ile, D-.Met, D-Ile, Orn, D-Orn
Leu	D-Leu, Val, D-Val, AdaA, AdaG, Leu, D-Leu, Met, D-Met
Pro	D-Pro, L-I-thioazolidine-4-carboxylic acid, D-or L-1-oxazolidine-4-carboxylic acid
Thr	D-Thr, Ser, D-Ser, allo-Thr, Met,D-Met, Met(O), D-Met(O), Val, D-Val
Ala	D-Ala, Gly, Aib, B-Ala, Acp, L-Cys, D-Cys
Val	D-Val, Leu, D-Leu, Ile, D-Ile, Met, D-Met, AdaA, AdaG
Gly	Ala, D-Ala, Pro, D-Pro, Aib, .beta.-Ala, Acp
Ile	D-Ile, Val, D-Val, AdaA, AdaG, Leu, D-Leu, Met, D-Met
Phe	D-Phe, Tyr, D-Thr, L-Dopa, His, D-His, Trp, D-Trp, Trans-3,4, or 5-phenylproline, AdaA, AdaG, cis-3,4, or 5-phenylproline, Bpa, D-Bpa
Tyr	D-Tyr, Phe, D-Phe, L-Dopa, His, D-His
Cys	D-Cys, S--Me--Cys, Met, D-Met, Thr, D-Thr
Gln	D-Gln, Asn, D-Asn, Glu, D-Glu, Asp, D-Asp
Asn	D-Asn, Asp, D-Asp, Glu, D-Glu, Gln, D-Gln
Lys	D-Lys, Arg, D-Arg, homo-Arg, D-homo-Arg, Met, D-Met, Ile, D-Ile, Orn, D-Orn
Asp	D-Asp, D-Asn, Asn, Glu, D-Glu, Gln, D-Gln
Glu	D-Glu, D-Asp, Asp, Asn, D-Asn, Gln, D-Gln
Met	D-Met, S--Me--Cys, Ile, D-Ile, Leu, D-Leu, Val, D-Val

TABLE III

Peptide name	Peptide sequence	Correspondance with human OX40L
P1 (SEQ ID NO:2)	VASLTYKDKVYLNVTTDNTSLDDFHVNGGEL	150-180
P2 (SEQ ID NO:3)	LDDFHVNGGELILIHQNPGEFCVL	160-183
P3 (SEQ ID NO:4)	VSHRYPRIQSIKVQFTEYKKEKGFILTSQ	52-80
P4 (SEQ ID NO:5)	EKGFILTSQKEDEIMKVQNNSVIINCDGFYL	72-102
P5 (SEQ ID NO:6)	IINCDGFYLISLKGYFSQEVNISLHYQKDEE	94-124
P6 (SEQ ID NO:7)	HYQKDEEPLFQLKKRSVNSLMVASLTYKDK	118-148
P5-1 (SEQ ID NO:8)	GYFSQEVNIS	107-116
P5-2 (SEQ ID NO:9)	ISLHYQKDEE	107-124
P5-3 (SEQ ID NO:10)	GFYLISLKGY	99-108
P5-4 (SEQ ID NO:11)	QEVNISLHYQ	111-120
P5-5 (SEQ ID NO:12)	IINCDGFYLI	94-103

REFERENCES

Al Shamkhani A et al., J Biol Chem, 272 (8):5275-5282, 1997.

Bodmer JL et al., Tr Bioch Sci, 27(1):19-26, 2002

Chen AJ et al., Immunity, 11 (6):689-698, 1999.

Cleland JL et al., Curr Opin Biotechnol,12: 212-9, 2001.

Cochran A et al., Curr Opin Chem Biol, 5 : 5^654-659, 2001.

Coleman RA et al., Drug Disc Today, 6(21) : 1116-1125, 2001.

Constans A, The Scientist, 16(4): 37, 2002

Dougherty DA, Curr Opin Chem Bio, 4: 645-52, 2000.

Golabek A et al., Biophys J, 79 (2):1008-1015, 2000.

Golebiowski A et al., Curr Opin Drug Discov Devel, 4: 428-34, 2001.

Gravestein L and Borst J, Semin Immunol, 10 (6):423-434, 1998.

Hruby VJ and Balse PM, Curr Med Chem, 7:945-70, 2000

Imura A et al., Blood 89 (8):2951-2958, 1997.

Kim HO and Kahn M, Comb Chem High Throughput Screen; 3: 167-8, 2000.

Kopf M et al., Immunity 11 (6):699-708, 1999.

Kraemer-Pecore CM et al., Cur Opin Chem Biol, 5(6):690-695, 2001.

Lowe CR et al., J Biochem Biophys Methods, 49(1-3):561-74, 2001.

Luo B and Prestwich GD, Exp Opin Ther Patents, 11: 1395-1410, 2001.

Murata K et al., J Exp Med, 191 (2):365-374, 2000.

Murphy LR et al., Protein Eng, 13:149-52, 2000.

Nohara C et al., J Immunol 166 (3):2108-2115, 2001.

Obinata M, Bioch Biophy Res Comm, 286 : 667-672, 2001

Pillai O and Panchagnula R, Cur Opin Chem Biol, 5: 447-451, 2001

Pippig SD et al., J Immunol 163 (12):6520-6529, 1999.

Ranney DF, Biochem Pharmacol, 59: 105-14, 2000.

Rogov SI and Nekrasov AN, Protein Eng, 14: 459-463, 2001

Sawyer TK, in "Structure Based Drug Design", edited by Veerapandian P, Marcel Dekker Inc., pg. 557-663, 1997.

Ullman E et al., Proc Natl Acad Sci, 91(12): 5426 – 5430, 1994.

Villain M et al., Chem Biol, 8(7):673-9, 2001

Weinberg A, Trends Immunology 23 (2):102-109, 2002.

CLAIMS

1. An OX40R binding agent selected from:
   a) polypeptides or peptides having the sequence corresponding to amino acids 94-124 (SEQ ID NO: 6), 107-116 (SEQ NO ID: 8), or 107-111 (SEQ ID NO: 13) of human OX40L;
   b) active mutants of the polypeptides or peptides of (a) in which one or more amino acid residues have been added, deleted, or substituted;
   c) peptide and non-peptide mimetics designed on the sequence and/or the structure of polypeptides or peptides of (a) or (b);
   d) polypeptides or peptides comprising the amino acid sequence of (a) or (b), and an amino acid sequence belonging to a protein sequence other than human OX40L (SEQ ID NO: 1);
   e) active fractions, precursors, salts, or derivatives of (a), (b), (c) or (d).
2. The OX40R binding agent of claim 1, wherein the polypeptide or peptide of (d) comprises the amino acid sequence belonging to one or more of these protein sequences: extracellular proteins, signal peptide-containing proteins, export signal-containing proteins, membrane-bound proteins, extracellular domains of membrane-bound protein, immunoglobulin constant region, multimerization domains,.
3. The OX40R binding agent of claim 1 or 2, wherein said binding agent is in the form of active conjugate or complex with a molecule chosen amongst radioactive labels, biotin, fluorescent labels, cytotoxic agents, drug delivery agents.
4. A nucleic acid segment which codes for a OX40R binding agent of claim 2.
5. A replicable vector of viral or plasmid origin which allows the expression of the OX40R binding agents encoded by the nucleic acid segment of claim 4.
6. A prokaryotic or eukaryotic host cell which has been modified to include the expression vector according to claim 5 and expresses a OX40R binding polypeptide of claim 2.
7. An isolated stable cell line substantially enriched in cells of claim 6.
8. The cell line of claim 7 wherein the OX40R binding agent is secreted.
9. The cell line of claim 7 wherein the OX40R binding agent is expressed on the membrane surface.

10. The cell line of claim 8 or 9 wherein the cells are human B cells.

11. A method of producing a OX40R binding agent according to claim 2 comprising culturing a host cell according to any of the claims 6 to 10 and collecting said binding agent.

12. Use of an OX40R binding agent according to any of claims from 1 to 3 as active ingredient in the manufacture of a pharmaceutical composition for the prophylaxis and/or treatment of diseases related to the activation of $CD4^+$ T cells.

13. A method for treating a patient suffering from a condition mediated by $CD4^+$ T cells comprising administering to the patient an effective amount of a OX40R binding agent according to any of the claims 1 to 3, or of a cell line according of any of the claims from 6 to 10.

14. Use of a OX40R binding agent according to any of the claims 1 to 3, or of cells according of any of the claims from 6 to 10 as agonist of human OX40L and stimulator of the activation of $CD4^+$ T cells expressing OX40R on their surface.

15. The use of claim 14 in the prophylaxis and/or treatment of transplantation rejection or cancer.

16. Use of a OX40R binding agent according to any of the claims 1 to 3, or of cells according of any of the claims from 6 to 10 as antagonist of human OX40L and inhibitor the activation of $CD4^+$ T cells expressing OX40R on their surface.

17. The use of claim 16 in the prophylaxis and /or treatment of autoimmune diseases, inflammation, or infection in a patient

18. The use of claim 16 for reducing the population of CD4' T-cells that express OX40R.

19. The use of any of the claims from 14 to 18 wherein the CD4+ T cell are comprised in a tissue sample from a patient and the method is carried out in vitro.

20. A pharmaceutical composition comprising a OX40R binding agent of any of the claims from 1 to 3 as active ingredient for the prophylaxis and/or treatment of diseases related to $CD4^+$ T cells.

21. The pharmaceutical composition of claim 19, in combination with a pharmaceutically acceptable carrier, excipient, stabilizer, or diluent.

22. The pharmaceutical composition of claim 14 or 15, wherein the diseases related to $CD4^+$ T cells are transplant rejection, autoimmune diseases, inflammation, infection, or cancer.

23. Means for modulating an immune response by altering OX40R-OX40L interaction *in vivo* or *ex vivo,* chosen amongst the agents of any of the claims from 1 to 3, the nucleic acids of claims 4 or 5, and the cells of any of the claims from 6 to 10.

24. Use of a OX40R binding agent according to any of the claims from 1 to 3 or of the cells according to any of the claims from 6 to 10 for the detection of the extracellular domain of OX40R protein as membrane-bound or a soluble protein.

25. Use of a OX40R binding agent according to any of the claims from 1 to 3 or of the cells according to any of the claims from 6 to 10 for the detection of activated $CD4^+$ T cells.

26. A method for the detection of the extracellular domain of OX40R protein, as membrane-bound or a soluble protein, in a sample, the method comprising:

a) contacting the sample with an OX40R binding agent according to any of the claims from 1 to 3 or with cells according to any of the claims from 6 to 10; and

b) detecting, directly or indirectly, the interaction between the extracellular domain of OX40R protein in the sample and the OX40R binding agent according to any of the claims from 1 to 3 or the cells according to any of the claims from 6 to 10 to indicate the presence of the extracellular domain of OX40R protein

27. A method of the detection of activated $CD4^+$ T cells in a sample the method comprising:

a) contacting the sample with an OX40R binding agent according to any of the claims from 1 to 3 or with cells according to any of the claims from 6 to 10; and

b) detecting, directly or indirectly, the interaction between the $CD4^+$ T cells in the sample and the OX40R binding agent according to any of the claims from 1 to 3 or the cells according to any of the claims from 6 to 10 to indicate the presence of the activated $CD4^+$ T cells.

28. Supports for the detection, the purification, and / or the concentration of OX40R extracellular domain, as membrane-bound or a soluble protein, or of activated $CD4^+$ T cells, said supports having OX40R binding agent according to any of the

claims from 1 to 3 or the cells according to any of the claims from 6 to 10 in an immobilized form.

29. Methods for the detection, the purification, and / or the concentration of OX40R extracellular domain, as membrane-bound or a soluble protein, or of activated $CD4^+$ T cells in a sample, said method comprising contacting said sample with the supports of claim 28.

30. A method of claim 26, 27, or 29, wherein the method is used to diagnose a condition associated to decreased or increased presence of $CD4^+$ T cells or of soluble OX40R protein.

31. A screening assay for the determination of the nature and the activity of compounds modulating OX40R-OX40L interactions comprising:

a) Forming a sample comprising the following elements:

i. An element constituting the OX40R binding agent, chosen amongst the compounds of any of the claims from 1 to 3, the cell line of any of the claims from 6 to 10, or the support of claim 27;

ii. An element constituting the OX40R moiety, chosen amongst a protein comprising the extracellular domain of OX40R, a cell line expressing OX40R extracellular domain on its surface, and a cell line secreting extracellular domain of OX40R; and

iii. The compound(s) to be tested as modulator(s) OX40R-OX40L interactions

b) Detecting, directly or indirectly, the effect of the compounds (iii) on the interactions between the elements (i) and (ii).

c) Comparing the effect detected in (b) amongst samples different in terms of quality and / or quantity of the elements of (a).

32. A kit for detecting extracellular domain of OX40R protein, as membrane-bound or a soluble protein, which comprises an OX40R binding agent according to any of the claims from 1 to 3, cells according to any of the claims from 6 to 10, or a support of claim 28.

33. A kit for detecting activated CD4* T-cells which comprises an OX40R binding agent according to any of the claims from 1 to 3, cells according to any of the claims from 6 to 10, or a support of claim 28.

34. A kit for the diagnosis of a condition due to a decreased or increased presence of $CD4^+$ T cells or of soluble OX40R protein in a sample obtained from a patient according to claim 32 or 33.

35. A kit for the determination of the nature and the activity of compounds inhibiting OX40R-OX40L interaction comprising an element chosen amongst the OX40R binding agent of claims 1 to 3, cells of any of the claims from 6 to 10, or a support of claim 28.

ABSTRACT

The present invention discloses peptides isolated from the extacellular domain of OX40 Ligand capable of binding OX40 Receptor and inhibiting OX40 Receptor - OX40 Ligand interaction. Such peptides, heterologous proteins comprising their sequences, as well as peptides and other molecules designed on their sequences, can be used as OX40 Receptor binding agents for modulating, as agonists or antagonists of OX40 Ligand, OX40 Receptor-mediated signaling in the prophylaxis and/or treatment of diseases related to activated T cells.

Figure 1

A)

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    Cytoplasmic domain         Transmembrane domain
<----------------------><------------------------------->
MERVQPLEEN VGNAARPRFE RNKLLLVASV IQGLGLLLCF TYICLHFSAL        50

                 Extracellular domain
<-----------------------------------------------------------
QVSHRYPRIQ SIKVQFTEYK KEKGFILTSQ KEDEIMKVQN NSVIINCDGF       100
 -------------P3----------------
                       -------------P4----------------
                                                --P5---
                 Extracellular domain
------------------------------------------------------------
YLISLKGYFS QEVNISLHYQ KDEEPLFQLK KVRSVNSLMV ASLTYKDKVY       150
                                           -----P1-----
P4
---------P5---------------
                 -------------P6------------------

         Extracellular domain
-------------------------------------->
LNVTTDNTSL DDFHVNGGEL ILIHQNPGEF CVL      183
--------P1-----------
         -----------P2-------------
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B)

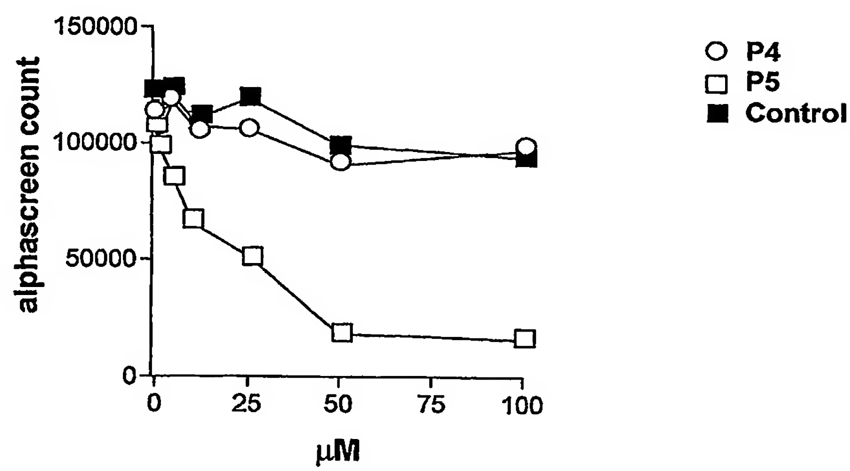


Figure 2

A)

```
P5      IINCDGFYLISLKGYFSQEVNISLHYQKDEE
P5-1                   GYFSQEVNIS
P5-2                            ISLHYQKDEE
P5-3          GFYLISLKGY
P5-4                         QEVNISLHYQ
P5-5    IINCDGFYLI
```

B)

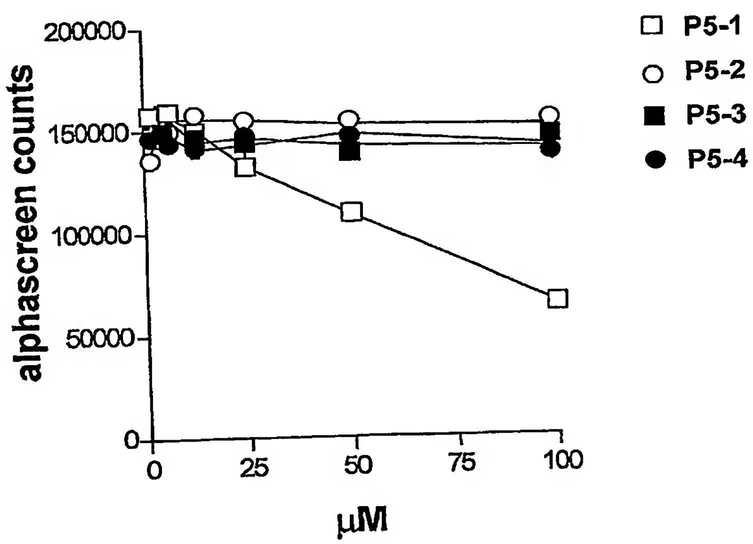
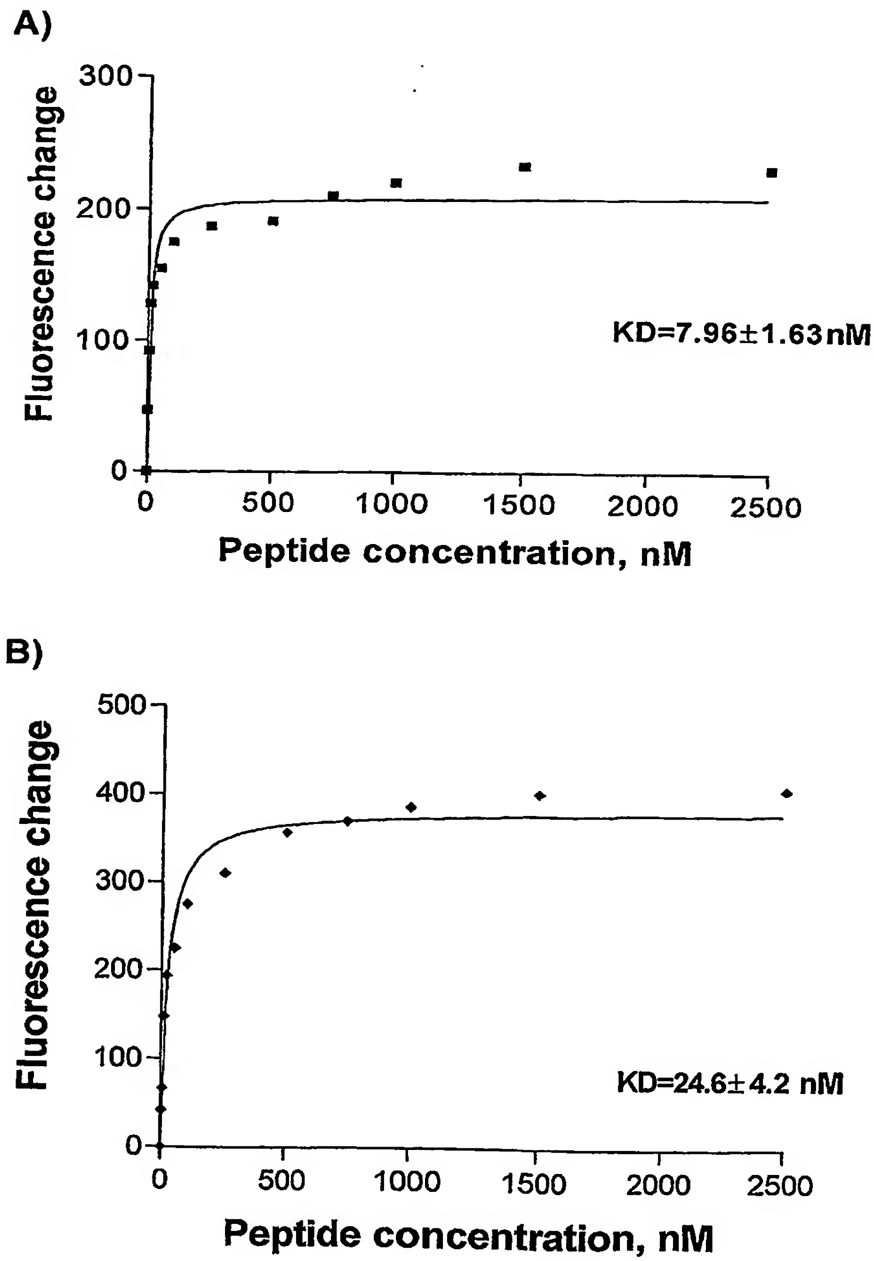


Figure 3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Fluorescence change
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200
100
0
0
500
1000
1500
2000
2500
Peptide concentration, nM
KD=7.96±1.63nM
B)
Fluorescence change
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100
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0
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Peptide concentration, nM
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Pro Arg Phe Glu Arg Asn Lys Leu Leu Leu Val Ala Ser Val Ile Gln
            20                  25                  30

Gly Leu Gly Leu Leu Leu Cys Phe Thr Tyr Ile Cys Leu His Phe Ser
        35                  40                  45

Ala Leu Gln Val Ser His Arg Tyr Pro Arg Ile Gln Ser Ile Lys Val
    50                  55                  60

Gln Phe Thr Glu Tyr Lys Lys Glu Lys Gly Phe Ile Leu Thr Ser Gln
65                  70                  75                  80

Lys Glu Asp Glu Ile Met Lys Val Gln Asn Asn Ser Val Ile Ile Asn
                85                  90                  95

Cys Asp Gly Phe Tyr Leu Ile Ser Leu Lys Gly Tyr Phe Ser Gln Glu
            100                 105                 110

Val Asn Ile Ser Leu His Tyr Gln Lys Asp Glu Glu Pro Leu Phe Gln
        115                 120                 125

Leu Lys Lys Val Arg Ser Val Asn Ser Leu Met Val Ala Ser Leu Thr
    130                 135                 140

Tyr Lys Asp Lys Val Tyr Leu Asn Val Thr Thr Asp Asn Thr Ser Leu
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Asp Asp Phe His Val Asn Gly Gly Glu Leu Ile Leu Ile His Gln Asn
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Pro Gly Glu Phe Cys Val Leu
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